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Human Cytomegalovirus Chemokine Receptor US28-induced Smooth Muscle Cell Migration Is Mediated by Focal Adhesion Kinase and Src*

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The human cytomegalovirus-encoded chemokine receptor US28 induces arterial smooth muscle cell (SMC) migration; however, the underlying mechanisms involved in this process are unclear. We have previously shown that US28-mediated SMC migration occurs by a ligand-dependent process that is sensitive to protein-tyrosine kinase inhibitors. We demonstrate here that US28 signals through the non-receptor protein-tyrosine kinases Src and focal adhesion kinase (FAK) and that this activity is necessary for US28-mediated SMC migration. In the presence of RANTES (regulated on activation normal T cell expressed and secreted), US28 stimulates the production of a FAK·Src kinase complex. Interestingly, Src co-immunoprecipitates with US28 in a ligand-dependent manner. This association occurs earlier than the formation of the FAK·Src kinase complex, suggesting that US28 activates Src before FAK. US28 binding to RANTES also promotes the formation of a Grb2·FAK complex, which is sensitive to treatment with the Src inhibitor PP2, further highlighting the critical role of Src in US28 activation of FAK. Human cytomegalovirus US28-mediated SMC migration is inhibited by treatment with PP2 and through the expression of either of two dominant negative inhibitors of FAK (F397Y and NH_2-terminal amino acids 1–401). These findings demonstrate that activation of FAK and Src plays a critical role in US28-mediated signaling and SMC migration.

Human cytomegalovirus (HCMV)[1] is a ubiquitous herpesvirus that establishes a life-long latent infection after the primary infection has been cleared. Although anti-viral therapy has appreciably reduced disease in transplant and AIDS patients, HCMV is still a significant problem in congenital disease and bone marrow transplant patients. In addition, HCMV has also been associated with long term diseases such as atherosclerosis, restenosis after angioplasty, chronic rejection after solid organ transplantation, and malignancies (1–4). The development of vascular disease involves a chronic inflammatory process with many contributing factors, and of these, chemokines and their receptors have been identified as key mediators. Interestingly, HCMV encodes a CXC chemokine (UL146), a potential CC chemokine (UL128), and four potential chemokine receptors (US27, US28, UL33, and UL78) with the most characterized being US28 (5–8). We have previously reported that US28 mediates arterial smooth muscle cell (SMC) migration and that this activity may contribute to viral dissemination and/or acceleration of vascular disease development (9).

US28 contains homology to the CC-chemokine receptors (10) and binds to a broad spectrum of chemokines including the CC chemokines RANTES, MCP-1, MCP-3, and MIP-1β and the CX_3C chemokine Fractalkine/CX3CL1 (11, 12). That CC chemokines fail to compete out Fractalkine binding suggests that Fractalkine binds to additional unique regions of US28 compared with the CC chemokines (11). In 293 cells, RANTES binding to US28 activates ERK1/2 pathways through the G-proteins $G\alpha_{i1}$ and $G\alpha_{16}$ (13). We have previously demonstrated that US28-mediated SMC migration also requires chemokine binding by either exogenously added RANTES or endogenously expressed MCP-1 (9). Induction of US28-mediated SMC migration is not blocked by treatment with pertussis toxin, a $G\alpha_{i/o}$ inhibitor, suggesting that other G-proteins are involved in this event. In fact, we have recently determined that US28 couples with $G\alpha_{12/13}$ to signal through RhoA.[2] This activity occurs in a ligand-dependent manner and is required for US28-mediated SMC migration. US28 has also been shown to exhibit constitutive signaling in COS-7 cells and human fibroblasts through both NF-κB and phospholipase C pathways via activation of $G_{q/11}$ G-proteins (14–16). Interestingly, Fractalkine binding to US28 as well as the US28 inverse agonist (VUF2274) but not CC chemokines antagonized constitutive signaling (15). Sequences have been identified in the US28 cytoplasmic domain that mediate signaling as well as recycling from the plasma membrane (17). Together these data highlight the fact that there are ligand-dependent activities and cell type-specific effects occurring in US28 signaling.

Lymphocyte migration induced by ligation of chemokine receptors is generally mediated through the $G\alpha_i$ class of hetero-

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[1] The abbreviations used are: HCMV, human cytomegalovirus; SMC, smooth muscle cell; MCP, monocyte chemoattractant protein; ERK, extracellular signal-regulated kinase; PTK, protein-tyrosine kinase; FAK, focal adhesion kinase; HA, hemagglutinin; m.o.i., multiplicity of infection; WT, wild type; RANTES, regulated on activation normal T cell expressed and secreted; NT, NH_2-terminal (1–410 amino acids); PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; MIP, macrophage inflammatory protein.

[2] R. Melnychuk, unpublished results.

trimeric G-proteins (18). Upon GPCR stimulation, G-proteins bind directly to the receptor, exchanging GDP for GTP, causing dissociation of the Gα subunit from the G$\beta\gamma$ complex. Downstream effectors of the activated Gα subunit are specific for each type of Gα subunit and can include Ca^{+2} influx, cAMP, and phosphatidylinositol phosphate. The G$\beta\gamma$ heterodimer activates signaling cascades other than those stimulated by the Gα subunit, which can include the protein-tyrosine kinase (PTK) pathways (19). For example ligand binding to CCR2, CCR5, and CXCR1/2 induce cellular migration through the PTK pathway (20–23). Activation of PTK(s) including that of focal adhesion kinase (FAK) is considered central for cellular migration. The importance of FAK in cellular migration was demonstrated by the inability of fibroblasts isolated from FAK−/− mice to migrate in response to stimuli (24). In addition, overexpression of FAK was shown to enhance movement of CHO cells (25), and highly invasive tumors characteristically have heightened levels of FAK expression and activity (26–29).

FAK is comprised of a central kinase domain flanked on one side by an N-terminal FERM (erythrocyte band 4.1-ezrin-radixin-moesin) domain, which is involved in linking FAK to integrins and/or growth factor receptors (30). The focal adhesion targeting domain is located C-terminal of the central kinase domain and is comprised of multiple protein-protein interaction motifs. The binding of paxillin and talin to the FAK-FAT domain facilitates a linkage to the cytoplasmic domain of integrins, which targets FAK to focal adhesions. FAK tyrosine phosphorylation after integrin or growth factor stimulation of cells is enhanced by its association with Src-family PTKs. This leads to the formation of a multiprotein signaling complex in which FAK serves as a scaffold. Mechanistically, after cell stimulation, FAK autophosphorylates at Tyr-397, resulting in the formation of an SH2 docking site. Src-family PTKs bind to FAK at Tyr-397, become activated, and trans-phosphorylate FAK at several other tyrosines including Tyr-925. The SH2 domain of the adaptor protein Grb2 binds to FAK Tyr-925 and forms a signaling complex that includes the nucleotide exchange factor Sos and the small GTP-binding protein Ras. This sequence of events contributes to the subsequent activation of ERK2/mitogen-activated protein kinase.

Previously, we have reported that treatment of SMC with the PTK inhibitors genistein or herbimycin A blocked US28-induced cellular migration, suggesting that PTK activity is required for migration of these cells (9). Here we demonstrate that US28 can signal through the non-receptor protein-tyrosine kinases Src and FAK and that this activity is required for the ability of US28 to mediate SMC migration. US28 activation of FAK occurs in a ligand-dependent manner, which is contrary to other reports suggesting that US28 may be constitutively active. In addition, we have determined critical regions of FAK that are required for US28-induced signaling and migration. These findings demonstrate that FAK and Src play integral roles in ligand-dependent, US28-mediated signaling associated with the induction of SMC migration.

MATERIALS AND METHODS

Cell Lines and Antibodies—The life-extended human pulmonary artery smooth muscle cell line, PAT1[2] was maintained in Medium 199 supplemented with 20% fetal calf serum and penicillin-streptomycin-L-glutamine (Invitrogen). For migration and *in vitro* kinase experiments described below PAT1 cells were utilized between passage 5 and 30 post-telomerization. Mouse FAK−/− fibroblasts were maintained on gelatin-coated culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin-streptomycin-L-glutamine, nonessential amino acids (Cellgro), and G418 (Sigma; 500 μg/ml) as previously described (24, 31). FAK−/− cells were used in experiments between passage 5 and 15.

Anti-Grb2 (C-7), anti-c-Src, anti-phosphotyrosine (PY99), and anti-HA (F-7) monoclonal antibodies were purchased from Santa Cruz Biotechnology. Phospho-specific antibodies to ERK2 (Thr-202/Tyr-204) and total ERK2 were from Cell Signaling Technologies. Paxillin antibodies (5H11) were from Upstate Biotechnologies, and anti-c-Myc tag antibodies were from Covance. The anti-FLAG (M2) monoclonal antibody was from Sigma and the anti-FAK polyclonal serum was previously described (31).

FAK in Vitro Kinase Assays—To determine whether US28 promotes the formation of an active FAK kinase complex we performed *in vitro* kinase assays on immunoprecipitated FAK from human SMC (30, 31). PAT1 SMCs were plated in 10-cm culture dishes and serum-starved for 24 h. The cells were co-infected with Ad-US28 and/or Ad-Trans at multiplicity of infection (m.o.i.) 500. After 16 h the cells were stimulated with RANTES (50 ng/ml) and then harvested at times 0 (unstimulated), 5, 10, and 15 min post-ligand addition. Cells were lysed in radioimmune precipitation assay buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, and total FAK was immunoprecipitated using rabbit anti-FAK polyclonal serum and protein A/G-conjugated agarose beads (Santa Cruz). Precipitation reactions were washed 1 time in radioimmune precipitation assay buffer, 2 times in HNTG buffer (50 mM HEPES, 150 mM NaCl, 1% Triton, 10% glycerol, pH 7.4), and 2 times in kinase buffer (20 mM HEPES, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 150 mM NaCl, 10% glycerol, pH 7.4) and then resuspended in kinase buffer plus [γ-^{32}P]ATP. The kinase reaction was allowed to proceed for 15 min at 32 °C and then analyzed by SDS-PAGE and autoradiography. The blots were analyzed by Western blotting for the presence of FAK and Src.

Immunoprecipitation Reactions—FAK−/− cells were plated in 10-cm culture dishes and serum-starved for 6 h upon 75% confluence. The cells were co-infected with Ad-Trans and/or Ad-US28 and/or Ad-FAK WT or FAK mutants at m.o.i. 50. After 16 h the cells were stimulated with RANTES (40 ng/ml) and then harvested at times 0 (unstimulated), 5, 10, 15, and 30 min post-ligand addition. Cells were lysed in radioimmune precipitation lysis buffer, total Grb2 was immunoprecipitated, and samples were analyzed by Western blotting using antibodies directed against Tyr(P). Co-precipitation of FAK-HA was demonstrated by stripping the blots in buffer containing 0.1 M Tris, pH 6.8, 1% SDS, and 1% 2-mercaptoethanol and staining using antibodies directed against HA. Before immune-complex reactions, a total of 50 μl of cellular lysate was assayed by SDS-PAGE/Western blotting for the presence of input US28 and FAK using antibodies directed against the HA tag present on both recombinant proteins. US28 co-immunoprecipitation reactions with Src were done as described above except that we used the FLAG-tagged version of US28, and the blots were probed with rabbit polyclonal antibodies directed against c-Src.

SMC Migration Assay—Cell migration assays were performed as previously described (9). Briefly, cells were added to the upper well of a Transwell (12-mm diameter, 3.0-μm pore size, Costar Corning, Cambridge, MA) at 1×10^5 cells per well. Cells were serum-starved for 16–24 h. HCMV at m.o.i. 10 was added to the upper well for 2 h. After infection the inserts were washed and transferred to fresh 12-well plates. Cells migrating to the lower chamber were counted at 48–72 h post-infection using a Nikon TE300 microscope at magnification 10×. Experiments were done in at least triplicate wells. Ten random fields were read in each well. The average number of cells per well was determined by multiplying the average number of cells per 10× field by the number of fields per well. Mean and S.D. were calculated. PP2 (1–25 μM; Calbiochem) was added 4 h after infection with HCMV to determine the role of Src in HCMV-US28-mediated SMC migration.

For SMC migration studies involving FAK dominant negative adenovirus constructs, SMC were infected with HCMV (m.o.i. 10) for 2 h followed by co-infection with Ad-Trans and Ad-FAK (WT, NT, Phe-397, Arg-454, or Pro-) at m.o.i. of 1000 for an additional 2 h. Subsequently, the Transwell were transferred to fresh 12-well plates. Cellular migration was determined as described above. Recombinant protein levels were monitored by Western blotting and equalized by adjusting the adenoviral vector m.o.i.

Adenovirus Construction—Adenoviruses expressing US28-FLAG were previously described (9). Adenovirus vectors expressing US28 with an N-terminal HA tag were constructed by subcloning the DNA fragment (14) into pAdTet7. This vector contains the tet-responsive enhancer within a minimal CMV promoter followed by the SV40 late poly(A) cassette, adenovirus E1A, and a single loxP site to increase recombination frequency. Recombinant adenoviruses were produced by pAdUS28-HA or pAdFAK (WT, NT, Pro, R454K, Phe-397) construct co-transfection of 293 cells expressing the Cre recombinase with adenovirus DNA (Ad5-ψ5) that contains an E1A/E3-deleted adenovirus genome (32). Recombinant adenoviruses were expanded on 293-Cre cells, and the bulk stocks were titered on 293 cells by limiting dilution.

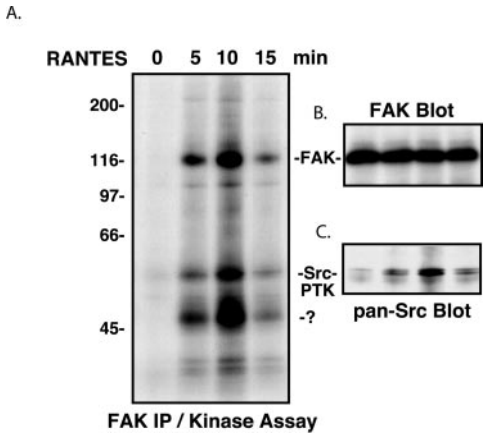

FIG. 1. **US28 activates FAK·Src complex by *in vitro* kinase assays.** SMC expressing US28 were untreated (0 time point) or treated with RANTES at 5, 10, and 15 min post-stimulation. Cells were harvested, and total FAK was immunoprecipitated (*IP*) (loading control shown in *B*) using anti-FAK antibodies. *A*, *in vitro* kinase assays were performed on FAK immunoprecipitation reactions. RANTES stimulation resulted in activating a FAK complex with pp60 Src between 5 and 10 min. *C*, Western blotting demonstrated that Src was associated with this FAK complex.

FAK and US28 expression were driven by co-infection with Ad-Trans expressing the Tet-off transactivator as previously described (9).

Immunocytochemistry—FAK−/− fibroblasts were grown in 0.1% gelatin-coated 4-well chamber slides (Nalge-Nunc). US28 and/or FAK was expressed using the adenovirus vectors described above. The cells were washed in phosphate-buffered saline, fixed in phosphate-buffered 1% paraformaldehyde for 10 min at room temperature, permeabilized, and blocked with 0.3% Triton X-100 in phosphate-buffered saline with 10% fetal calf serum and 0.1% sodium azide. Thereafter, the cells were incubated with antibodies against US28-FLAG epitope or FAK-HA epitope or FAK-c-Myc epitope (FAK-NT mutant only) in a 1:200 dilution for 1 h at room temperature. Cells were washed three times in phosphate-buffered saline, and binding of the primary antibody was detected with a fluorescein isothiocyanate tetramethyl-conjugated goat anti-mouse or rhodamine-conjugated goat anti-rabbit antibody for 1 h at room temperature. At this time the cells were also stained for actin using phalloidin (Molecular Probes, Eugene, OR) to monitor alterations in cellular actin cytoskeleton induced by US28 and FAK. Fluorescence-positive cells were visualized on an inverted Nikon fluorescent microscope.

RESULTS

US28 Activation of FAK and Src Is Ligand-dependent—We have previously demonstrated that the HCMV-encoded chemokine receptor US28 mediates arterial SMC migration (9). Migration occurred in a ligand-dependent manner that was sensitive to PTK inhibitors, suggesting a role for PTK activation in US28-mediated SMC migration. FAK is a non-receptor PTK with critical importance in facilitating cellular migration. To determine whether FAK plays a role in US28-mediated SMC migration, we first determined whether US28 ligation with RANTES promoted the activation of FAK. For these experiments, we performed *in vitro* kinase assays on FAK immunoprecipitation reactions. Adenovirus vectors were used to express HCMV-US28 in PAT1 SMC using methods that have been previously described (9). Serum-starved US28-expressing PAT1 cells were untreated (0 time point) or treated with 50 ng/ml RANTES and harvested at 5, 10, or 15 min after the addition of ligand. Total cellular FAK was immunoprecipitated, and the samples were subjected to *in vitro* kinase assays and examined by SDS-PAGE and autoradiography. RANTES binding to US28 promoted the formation of a FAK·Src kinase complex by 5 min post-addition of ligand, and this activity was maximal at 10 min. FAK·Src kinase activity was reduced by 15 min, suggesting that the complex is transient (Fig. 1). PAT1 cells not expressing US28 failed to promote the FAK·Src kinase complex in the presence of RANTES (data not shown). Untreated US28-expressing PAT1 cells (0 time point) failed to activate the FAK·Src kinase complex, suggesting that this event is not due to an inherent constitutive signaling property of US28 but, rather, requires ligand binding. Western blotting using antibodies directed against FAK showed equal FAK protein levels for each immunoprecipitated sample, and antibodies directed against Src confirmed the association of this protein-tyrosine kinase with FAK (Fig. 1). Interestingly, an additional ~47-kDa protein was co-immunoprecipitated with the FAK·Src complex, and this protein was highly phosphorylated, suggesting that RANTES binding to US28 facilitates the formation of a FAK and Src kinase complex, which includes additional signaling molecules.

US28 Forms a Protein Complex with Src—Previously we demonstrated that US28-mediated SMC migration is blocked by treatment with the pan-PTK inhibitors genistein and herbimycin A (9). Above we show that US28 activates a FAK·Src kinase complex, suggesting that Src plays an important role in US28/FAK signaling. To determine whether Src activity was required for US28-mediated SMC migration, we performed migration assays in the presence of increasing concentrations of PP2. As shown in Fig. 2*A*, treatment of SMC expressing US28 with PP2 resulted in near total inhibition of cellular migration, which occurred in a dose-dependent manner. We next examined the mechanism involved in US28 activation of Src. We next determined whether US28 complexes with Src by co-immunoprecipitation assays using antibodies directed against the FLAG epitope-tagged version of US28. As shown in Fig. 2*B*, antibodies directed against US28-FLAG co-precipitated Src upon the addition of RANTES. The complex is formed by 2.5 min after stimulation and is maximal between 2.5 and 5 min. The US28 association with Src returns to near background levels by 10 min (Fig. 2*B*). The kinetics of Src/US28 association are similar to those observed for Src/β_2-adrenergic receptor binding after the addition of ligand (33). These findings suggest that US28 either binds directly to Src or the interaction is mediated through binding to other proteins like β-arrestin, as occurs with the β_2-adrenergic receptor.

US28 Mediates the Association of FAK with Adaptor Proteins in a Ligand-dependent Manner—Upon cellular activation by extracellular matrix proteins, Src phosphorylates FAK at multiple protein binding sites and increases the ability of FAK to bind adaptor proteins including paxillin and p190RhoGEF (focal adhesion targeting domain), Grb2 (Tyr-925), p130Cas (P*XX*P motif at 712 and 876), and GRAF (P*XX*P motif at 876). Binding of these adaptor proteins promotes signal transduction and the assembly of focal adhesions. Our findings above that US28 promotes the formation of a FAK·Src kinase complex that contains additional phosphorylated proteins suggests that US28 promotes the functional activation of FAK through the binding of adaptor proteins. To determine whether US28 induces the association of adaptor proteins with FAK we performed FAK·Grb2 co-immunoprecipitation reactions in FAK−/− cells expressing either US28-HA and/or WT FAK-HA. Cells were treated with RANTES and harvested at 0 (unstimulated), 5, 10, 15, or 30 min. Total cellular Grb2 was immunoprecipitated, and the blots were first stained for total tyrosine phosphorylation and then reprobed for FAK-HA. US28 promoted the association of FAK with Grb2 (Fig. 3*A*). Interestingly, only in the presence of FAK did US28 activate a highly Tyr-phosphorylated Grb2 complex, and FAK association with Grb2 similarly only occurred in the presence of US28. In the US28- and FAK-expressing cells there was a higher level of Tyr phosphorylation, suggesting that either these cells express low levels of US28 ligands (*i.e.* mouse MCP-1 is a ligand for US28)

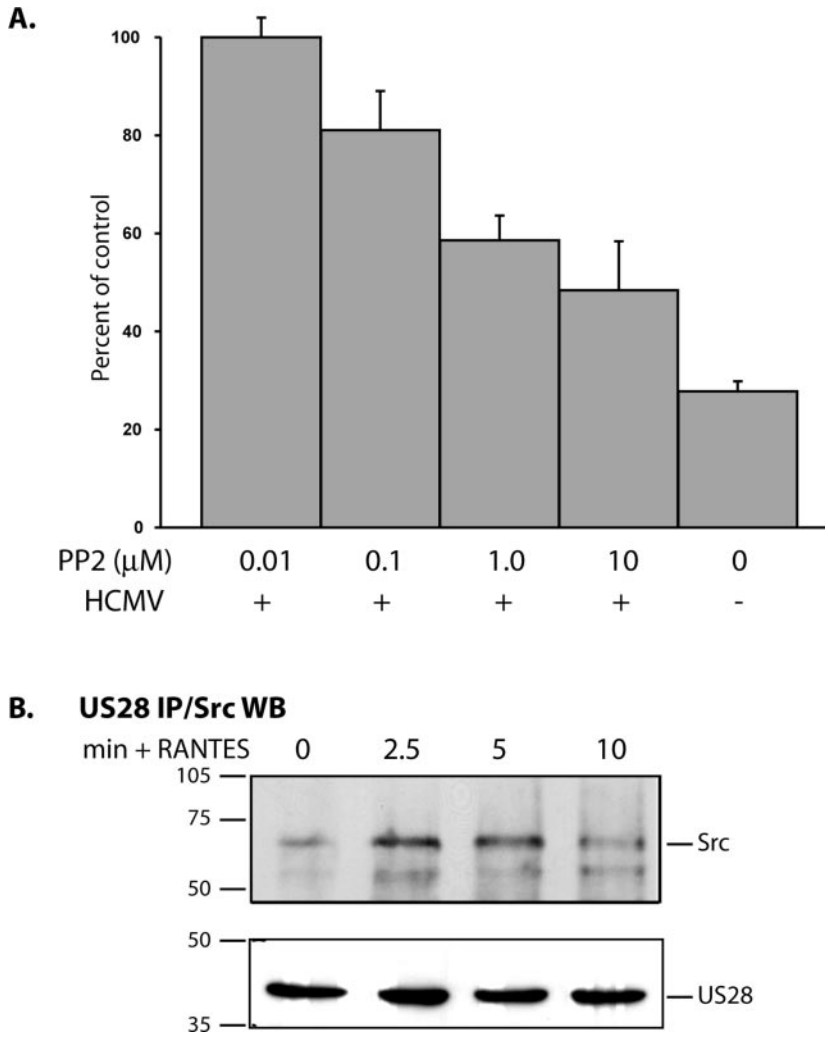

FIG. 2. **US28 binds Src and PP2 blocks US28-mediated SMC migration.** *A*, HCMV-US28 SMC migration assays were performed in the presence of increasing concentrations of the Src inhibitor PP2. *B*, US28 was immunoprecipitated (*IP*) from FAK−/− fibroblasts using antibodies directed against the FLAG epitope at 0 (unstimulated), 2.5, 5, and 10 min post-addition of RANTES. Western blotting demonstrated equal US28 loading.

or that this is due to US28 constitutive activity. Interestingly, this effect is only present in the presence of FAK. The US28 promotion of Grb2·FAK complex was maximal between 5 and 15 min post-addition of RANTES (Fig. 3*A*), which displays similar kinetics as US28 activation of the FAK·Src kinase complex described in Fig. 1*A* but follows the activation of Src as shown in Fig. 2*B*. Together these findings demonstrate that US28 activation of FAK·Src kinase complex is functional and promotes the binding of adaptor proteins to FAK.

To determine whether US28 activation of Src was critical for FAK activation, we analyzed the regulation of FAK activation by US28 in the presence of the Src-specific inhibitor PP2 using Grb2·FAK co-immunoprecipitation assays as described above. Treatment with PP2 for 1 h before the addition of RANTES blocked the ability of US28 to induce functional activation of FAK (Fig. 3*C*). At a concentration of 33 μM, PP2 reduced the level of FAK binding to Grb2 to background levels of non-stimulated US28-expressing cells. Together these findings suggest that US28 activates Src and that this activity is required for the ability of US28 to activate FAK and induce SMC migration.

US28 mediates the activation of ERK1/2 through the $G\alpha_{i1}$ and $G\alpha_{16}$ G-proteins in a ligand dependent manner (13). FAK is also an important regulator of ERK activity through its association with Grb2/SOS. To determine whether US28 activation of ERK required FAK for signal transduction, we used Western blotting to assay the status of ERK1/2 phosphorylation in FAK-null cells in the presence of US28 with and without FAK reconstitution. For these experiments FAK null cells expressing US28-HA, FAK-HA, or US28-HA and FAK-HA were treated with RANTES and harvested at 0 (unstimulated), 5, 10, and 15 min after the addition of ligand. US28 activation of ERK was ligand-dependent and occurred in the US28/FAK-expressing cells by 5–10 min and sustained for 15 min (Fig. 4, *A* and *B*). However, in the absence of FAK, RANTES binding to US28 failed to activate ERK, but this activation was delayed (10 min *versus* 5 min), and the levels of ERK phosphorylation were dramatically reduced compared with cells expressing US28 in the presence of FAK. This finding of FAK-independent activation of ERK is similar to that observed for growth factor and integrin stimulation where activation of ERK is reduced (34). Nonetheless the ability of US28 to activate ERK2 occurs in a ligand- and FAK-dependent manner with kinetics that are similar to the formation of the FAK·Grb2 complex that is induced by US28.

US28-mediated Cytoskeleton Rearrangements and SMC Migration Are Dependent upon FAK Activity—FAK is comprised of a central kinase active core flanked by multiple domains that link FAK to the cytoskeleton and promote interactions with other adaptor proteins. Thus FAK is a scaffold for signal transduction. To identify which domains of FAK are required to transmit US28 signal transduction and mediate SMC migration, we constructed adenoviruses expressing WT FAK and dominant negative FAK mutants (F397Y autophosphorylation/SRC homology SH2 binding site mutant, R454K kinase inactive, Pro-deleted of the proline rich regions that bind adaptor proteins, and NT containing only the first 401 amino acids of FAK). Fig. 5 shows the various mutants and their cellular

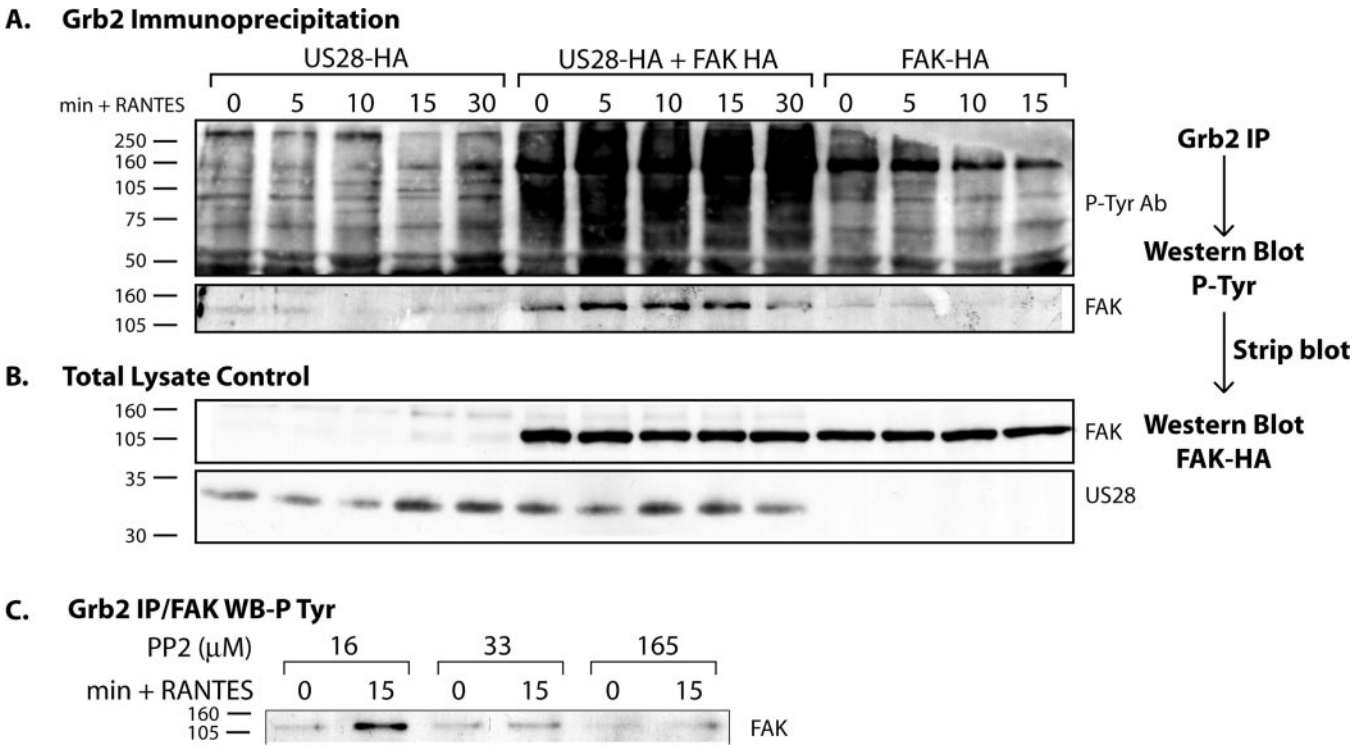

FIG. 3. **US28 induces FAK·Grb2 complex formation.** *A*, to determine whether US28 promotes the association of FAK with the adaptor protein Grb2, co-immunoprecipitation reactions were performed. FAK−/− cells expressing US28, WT FAK, or US28 and WT FAK were stimulated with RANTES and harvested at 0 (unstimulated), 5, 10, 15, 30 min post-addition of ligand. Cells were lysed in a modified radioimmune precipitation lysis buffer, and total cellular Grb2 was immunoprecipitated (*IP*) using specific antibodies and protein A/G-conjugated Sepharose beads. Samples were analyzed by SDS-PAGE and Western blotting (*WB*) first for Tyr(P) (*P-Tyr*) and then FAK-HA. *B*, Western blot demonstrating equal expression of FAK and US28. *C*, to determine whether Src activity was required for US28-mediated activation of FAK, US28 expressing FAK-reconstituted FAK null cells were treated with increasing concentrations of PP2 before activation with RANTES. At 0 (unstimulated) and 10 min post-RANTES addition, samples were harvested, and Grb2·FAK coimmunoprecipitation reactions using antibodies to Grb2 were performed as described above. The samples were analyzed by SDS-PAGE and Western blotting using antibodies directed against FAK-HA tag.

localization. All of the FAK constructs localized to focal adhesions except FAK-NT, which demonstrated a disperse cytoplasmic-staining pattern.

US28 expression in FAK null cells failed to induce the formation of Tyr-phosphorylated Grb2 complexes (Fig. 3*A*), suggesting that the ability of US28 to signal through these adaptor proteins requires FAK as a scaffold. FAK−/− cells have a characteristic actin morphology that lacks a coordinated network of actin stress fibers, which is overcome through reconstitution of FAK and stimulation by extracellular matrix proteins or constitutively active vSrc (32). Expression of US28 in FAK−/− cells did not alter the actin morphology in these cells even in the presence of ligand (Fig. 6*A*). These results confirm the role of FAK in US28 signaling. Reconstitution of the FAK−/− cells expressing US28 with WT FAK induced the rearrangements in the actin cytoskeleton through the assembly of actin stress fibers (Fig. 6*B*). This effect on actin morphology was enhanced when stimulated for 2 h with RANTES (Fig. 6*B*) and was absent in cells not expressing US28 (data not shown). This suggests that activation of US28 promotes FAK-dependent cytoskeleton rearrangements, which resemble the changes in actin morphology observed in FAK reconstituted FAK−/− cells expressing vSrc (32). Reconstitution with FAK-Arg-454 (kinase inactive mutant) displayed similar effects on the cellular actin structure compared with WT FAK (Fig. 6*E*), suggesting that the kinase activity of FAK is not required for this process. However, FAK-Phe-397 and FAK-NT failed to affect the actin arrangement in the cells even in the presence of ligand (Fig. 6, *D* and *F*). FAK-Pro displayed an intermediate effect on actin morphogenesis that was only visible in the presence of the US28 ligand (Fig. 6*C*). Together these findings suggest that FAK is required in part for the ability of US28 to mediate actin rearrangements that are associated with cellular migration. FAK activation through the autophosphorylation/Src binding site at Tyr-397 is critical for this activity but not sufficient since the FAK NT mutant containing this site fails to promote US28 morphological changes, presumably because it lacks the FAT domain and, thus, the ability to interact with the cytoskeleton and mediate signal transduction.

To determine whether focal adhesion kinase activity was necessary for US28-mediated SMC migration, HCMV-infected SMC were co-infected with adenoviruses expressing dominant negative FAK mutants (Phe-397, R454K, Pro, or the NT mutant). FAK protein expression in PAT1 SMC was determined by Western blotting and then equalized by adjusting the adenovirus m.o.i. HCMV-US28 migration assays were performed as previously described (9). HCMV-mediated SMC migration was inhibited by the Phe-397 and NT FAK mutants, consistent with their lack of ability to induce actin rearrangements associated with US28 in the presence of functional FAK (Fig. 7, *A* and *B*). Thus, US28-induced SMC migration requires the autophosphorylation activity and/or downstream effects that are associated with FAK. Interestingly, expression of WT FAK or FAK-Arg-454 consistently enhanced HCMV-US28-mediated SMC migration (Fig. 7, *A* and *B*), similar to the findings of Cary *et al.* (25) wherein FAK overexpression in CHO cells increased their capacity to migrate. Together with the above observations that US28 mediates cellular cytoskeleton rearrangements through FAK, these data suggest that the mutant lacking

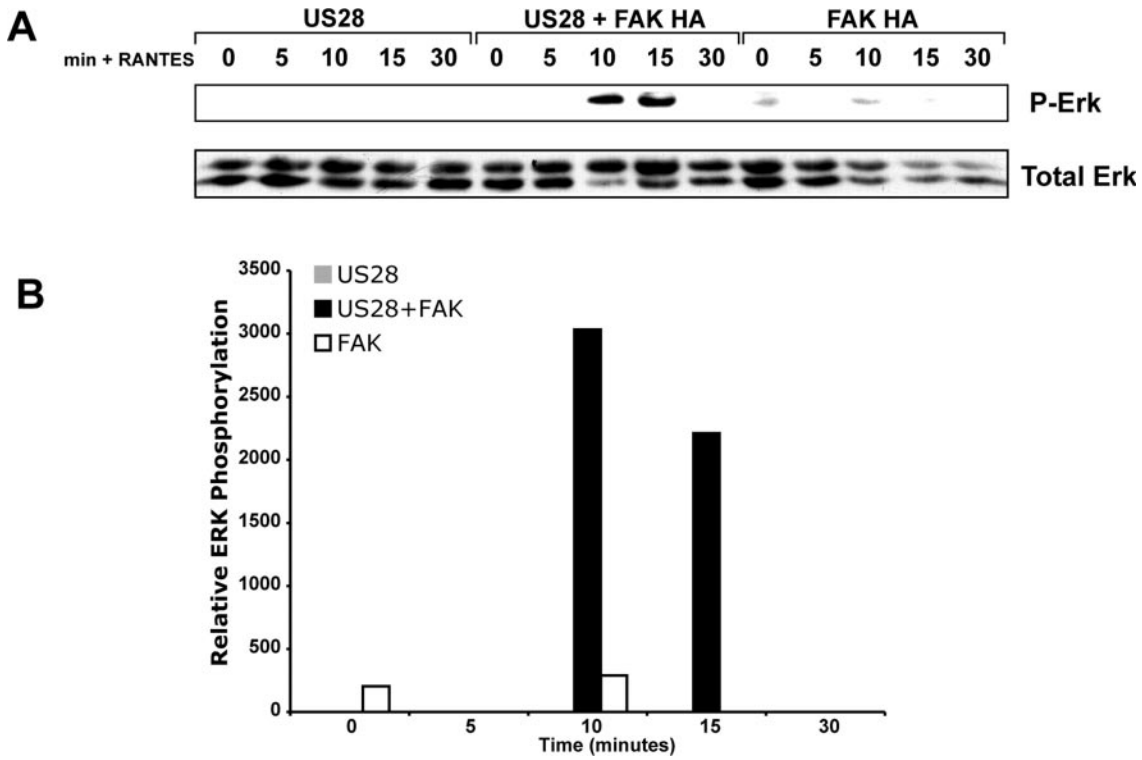

FIG. 4. **US28 activation of ERK requires FAK.** To determine whether US28 activation of ERK requires FAK, we performed Western blot analysis for active ERK using phospho (*P*)-specific ERK antibodies. FAK null cells expressing either US28 alone, WT FAK alone, or US28 and WT FAK were stimulated with RANTES and harvested at 0 (unstimulated), 5, 10, 15, and 30 min post-addition of ligand. To demonstrate equal ERK loading, the above blots were reprobed for total ERK.

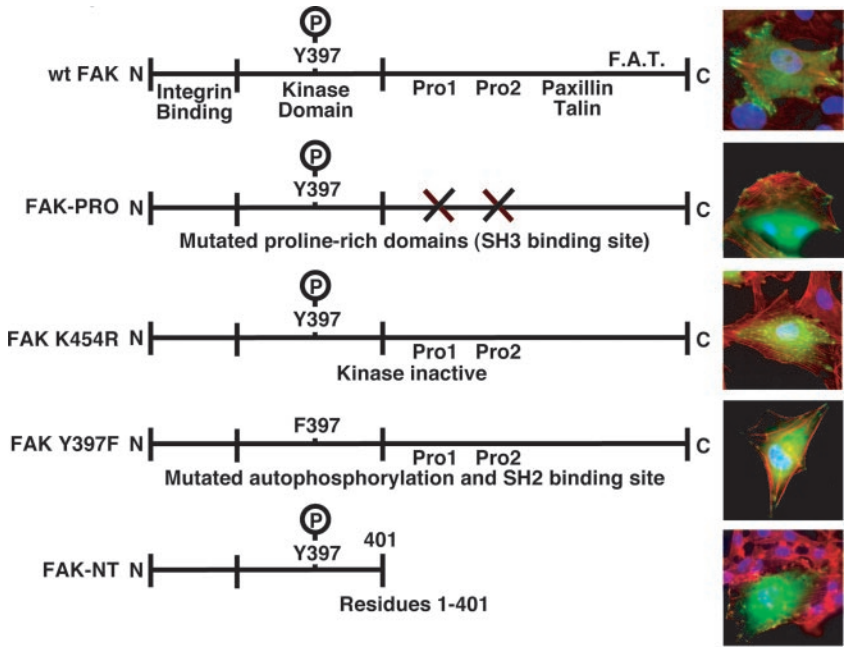

FIG. 5. **Construction of adenovirus vectors expressing FAK mutants.** Shown is a schematic map and immunofluorescence analysis of WT FAK and the FAK mutants (Phe-397, NT, Arg-454, and Pro). FAK-expressing cells were stained with antibodies directed against the FAK-N-terminal HA tag (*green*) or N-terminal C-Myc tag (*green*; FAK-NT), actin was stained with phalloidin (red), and nuclei were stained with Hoescht dye (*blue*). *F.A.T.*, focal adhesion targeting; *P-*, phosphorylated.

kinase activity can still function with wild-type properties. The FAK-Pro caused an intermediate phenotype as overexpression of this mutant did not block SMC migration nor did it enhance migration. This intermediate effect was similar to the effect of this mutant on promoting alterations in cellular morphology. Overall our findings demonstrate an important role for FAK and Src in ligand-dependent, US28-mediated signaling and SMC migration.

DISCUSSION

In this study, we demonstrate that US28 signals through the non-receptor tyrosine kinases FAK and Src in a ligand-dependent manner and that this activity has a direct role in HCMV-mediated SMC migration. These observations suggest a model (Fig. 8) whereby ligand binding to the HCMV-US28 chemokine receptor binds and activates Src, which then promotes FAK activation through the phosphorylation of critical sites on FAK

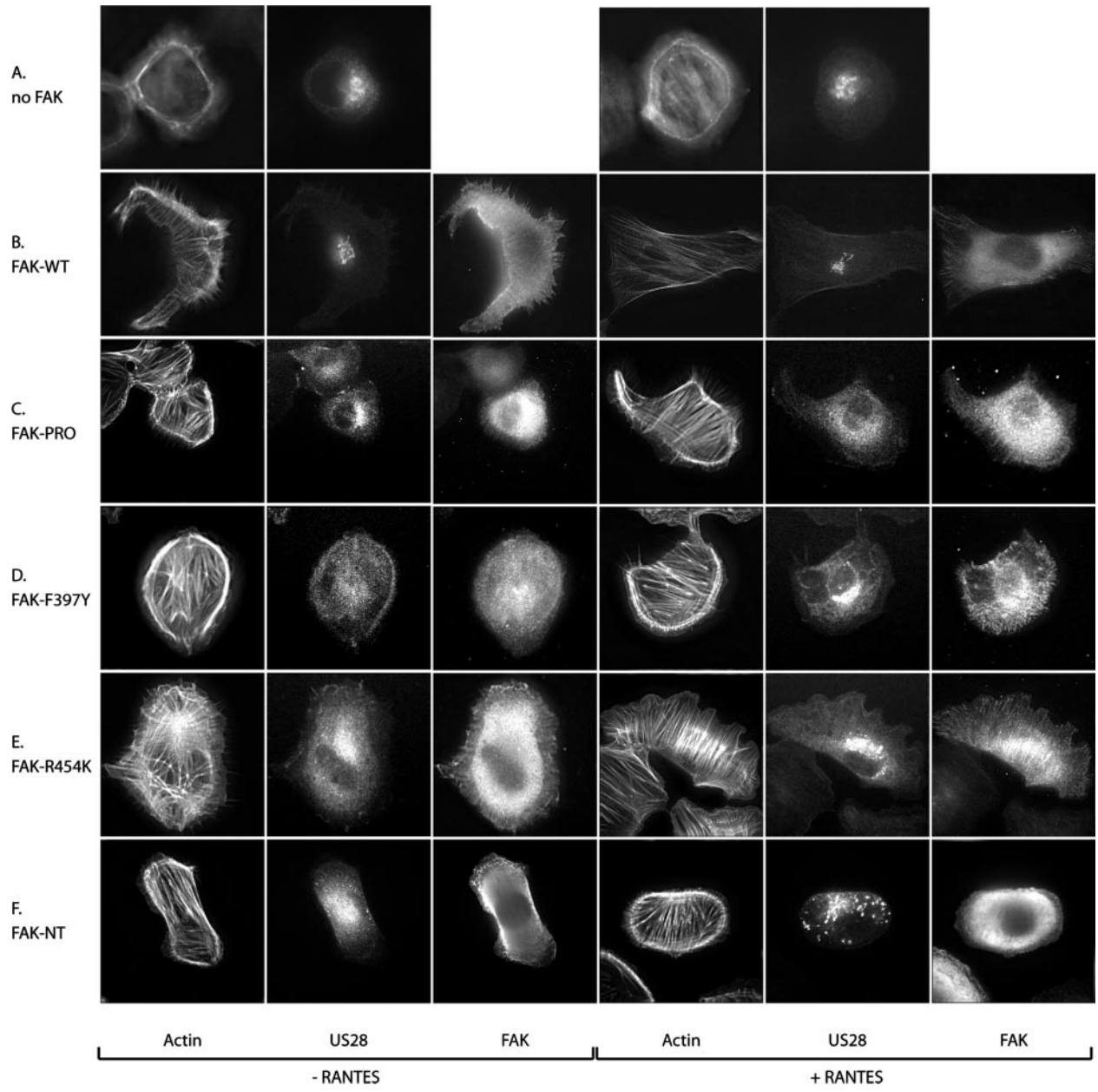

FIG. 6. **US28-induced actin stress fiber formation is FAK-dependent.** FAK null cells expressing US28 were lacking FAK (*A*) or reconstituted with WT FAK (*B*) or the FAK mutants Pro (*C*), Phe-397 (*D*), Arg-454 (*E*), or NT (*F*). RANTES-treated cells were fixed 2 h post-addition of ligand. Cells were stained for actin with phalloidin, for FAK using antibodies directed against the FAK-N-terminal HA tag or c-Myc tag (NT-FAK only), and for US28 using antibodies directed against the N-terminal FLAG epitope present on US28.

that mediate protein-protein interactions with adaptor proteins such as Grb2. Activation of Grb2/Sos promotes the activation of ERK1/2 through RAS, which is important to initiate transcriptional events necessary for cellular migration. In the FAK-reconstituted FAK−/− cells we observed that US28 signaling induced the rearrangement of the actin cytoskeleton, which is necessary for the induction of migration. Thus, FAK and Src play a central role in US28-mediated signaling and migration through promotion of focal adhesion formation/disassembly and cytoskeleton rearrangements that are critical for motility.

US28 Activation of Src and FAK—US28 can signal in response to ligand binding and also in the absence of the addition of exogenous ligands. Interestingly, the types of signaling pathways that are evoked in the presence or absence of US28 ligands are dramatically different. Physiological relevant pathways need to be used in functional assays to determine the relevance of the signaling observed in constitutive *versus* ligand-dependent pathways. Although constitutive US28 signaling may occur during the life cycle of HCMV infection, a physiological role for this event has not been identified. However, our studies indicate that US28 ligand binding induces SMC migration, which is dependent upon the signaling events involved in this process.

Inhibition of Src with PP2 abrogated US28-mediated SMC migration, suggesting a functional relationship of Src in US28-mediated SMC migration. US28 activation of Src occurs before that of FAK since the US28 Src interaction occurred before the activation of FAK and since inhibition of Src blocked FAK association with Grb2. A number of mechanisms have been implicated in the activation of Src by GPCRs. However, currently ligand binding to a GPCR is considered to promote the release of the G-protein $\beta\gamma$ subunits, which then target G-protein-coupled receptor kinases to the activated receptor.

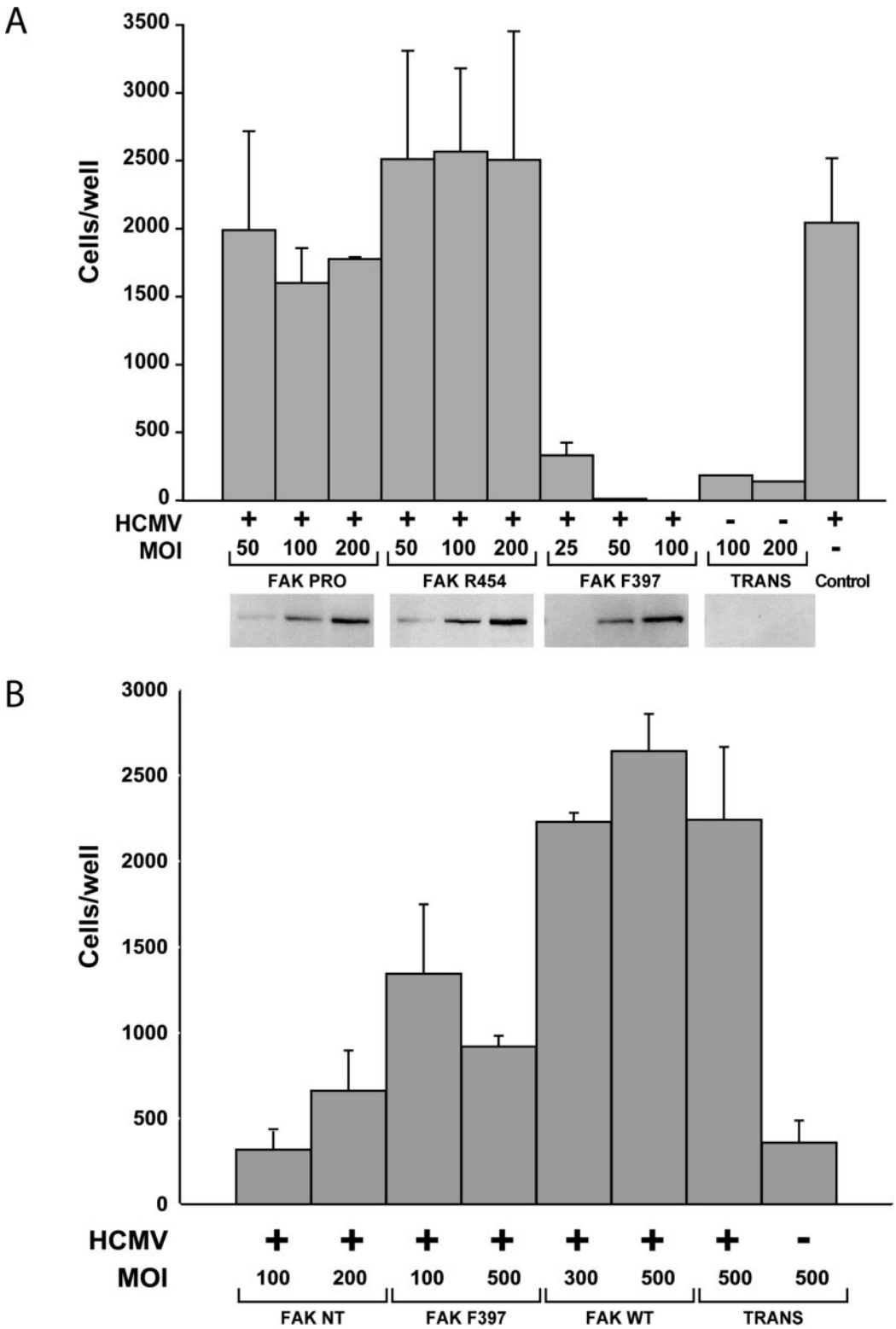

FIG. 7. **FAK activity is required for US28-mediated SMC migration.** To determine whether FAK activity was necessary for US28-mediated SMC migration, HCMV-infected SMC were co-infected with adenoviruses expressing dominant negative FAK mutants (Phe-397, R454K, and Pro in *A* or WT FAK, Phe-397, and NT in *B*). HCMV-mediated SMC migration was inhibited by the NT and Phe-397 mutants. FAK mutant protein expression was determined by Western blotting using antibodies directed against an N-terminal HA tag.

G-protein-coupled receptor kinases promote receptor internalization and the association of the GPCR with arrestins. Src is targeted to the active receptor-arrestin complex because they contain Src binding domains. Interestingly, we demonstrate here that US28 forms a complex with Src in the presence of RANTES, which may occur via a direct interaction or through binding of adaptor proteins like β-arrestin. However, two independent groups have provided conflicting data with regard to the association of US28 with β-arrestin. One group has shown that US28 C-terminal tail sequences are responsible for the interaction of US28 and G-protein-coupled receptor kinases and/or β-arrestin and they also demonstrate the importance of this region in ligand-independent signaling through phosphatidylinositol 1,4,5-trisphosphate and p38 (35). However, a differ-

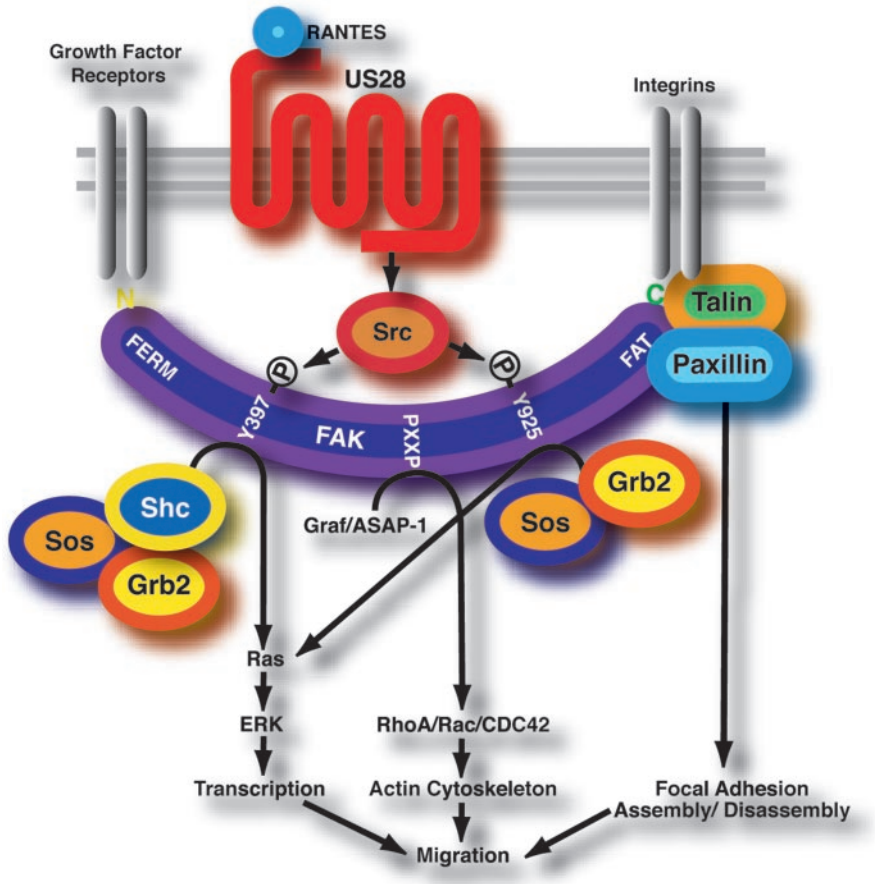

FIG. 8. **Model of FAK and Src in US28-mediated signaling and SMC migration.** RANTES binding to US28 promotes interactions with and activation of Src, which then mediates the formation of an active FAK·Src kinase complex. Src phosphorylates FAK at a number of sites including the Grb2 binding domain (Tyr-925), which promotes the activation of Grb2/Sos. Thus, FAK acts as a signaling scaffold to promote US28-mediated SMC migration.

ent report demonstrates that US28 internalization is not altered in β-arrestin−/− fibroblasts, although this second study failed to determine whether US28 signaling was altered in these cells (36). Thus, the mechanisms of ligand-dependent US28 activation of Src require further investigation.

RANTES binding to the cellular CC-chemokine receptor CCR5 is also known to activate FAK in T-cells. Interestingly, AOP-RANTES, a derivative of RANTES that binds CCR5 with similar kinetics as RANTES, does not induce T-cell migration and failed to activate FAK, indicating that FAK activation is important for cellular migration (22). Here we demonstrate that US28 activates FAK upon RANTES binding. Activation of FAK was crucial for the induction of SMC migration as two independent mutants of FAK (F397Y and NT) were capable of blocking US28-induced motility. This is the first demonstration that expression of the FAK FERM domain (FAK-NT) can act as a dominant inhibitor of migration. The FAK-F397Y mutant is capable of localizing to focal adhesions but fails to interact with Src, rendering this FAK molecule unable to become activated or transduce signals. The mechanism of the FAK-NT mutant in blocking US28-mediated SMC migration is not fully understood but may work by sequestering active Src because it contains a natural autophophorylation/SH2 binding domain (30). These results demonstrate the critical role of FAK activation in US28 mediated signaling events that occur before the induction of migration.

US28 in the Signaling and Migration Processes Associated with Disease—The ability of US28 to mediate cellular migration distinguishes this GPCR from other virus-encoded chemokine receptors. Previously, we demonstrated that US28 mediates the migration of arterial SMC. This migration may have important implications in the development of vascular diseases through the increased movement of these cells in and around vascular lesions. However, US28 may alter or enhance the migratory capacity of a number of other cell types yet to be explored. For instance, HCMV establishes latency in hematopoietic progenitor cells and monocytes, and these cells are subject to extensive chemokine binding potentials under inflammatory conditions. Interestingly, the promonocytic cell line THP-1 has been reported to express US28 in the absence of viral early or late genes, suggesting that US28 may be expressed during the non-productive phase of virus replication (37). If US28 is expressed in monocytes or progenitor cells, the virus may be able to direct or target these virus-infected cells to areas of local inflammation through US28 binding to RANTES, MCP-1, or even the endothelial cell membrane-bound chemokine Fractalkine. HCMV has acquired many different strategies of immune evasion; US28 expression in these cells may misdirect virus-infected monocytes to sites of inflammation instead of the lymph node structures involved in the normal antigen retrieval/presentation arm of the immune system. In HCMV biology this concept actually makes sense because inflammatory conditions promote reactivation and replication of this herpesvirus. Thus, targeting areas of inflammation by infected cells would be favorable for HCMV persistence. In support of this hypothesis, a number of studies indicate that deletion of the viral chemokine receptor homologues to HCMV-UL33 in mouse CMV and rat CMV, m33 and r33 respectively, results in the lack of virus replication in the host salivary glands (38, 39). The viral GPCRs may mediate this process through expression in CMV-infected macrophages, which alters migration of these cells to different tissues.

Our studies suggest that one of the critical mechanisms involved in HCMV-US28-mediated SMC migration is the activation of the PTK focal adhesion kinase. Because US28 activation of FAK and SMC migration was sensitive to the drug PP2,

which is potent inhibitor of Src, and because US28 activates a FAK·Src kinase complex, it may be suggested that Src plays an important role in US28 activation of FAK. FAK and Src are important modulators in a number of cellular processes and are implicated in a number of disease states. Because HCMV is associated with vascular disease as well as tumors that are highly invasive, the linking of US28 to activation of FAK and Src may have significant implications in these diseases.

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